An Effect of Iron Level and Food Type on Production of Hemolysin and Catalase of *Listeria monocytogenes* Strain Scott A

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ABSTRACT

Virulence of *Listeria monocytogenes*, a foodborne pathogen, has been attributed in humans to the presence of hemolysins and oxidative enzymes that permit entrance and survival in macrophages and intestinal cells. Levels of hemolysin, catalase, and iron in sterile skim milk, meat, and various media inoculated and incubated under various conditions were determined. High-iron media and aeration increased growth and induced production of several catalases based on SDS-PAGE analysis. Reduced aeration increased hemolysin activity, but growth and catalase production were reduced. At refrigerator temperatures, *L. monocytogenes* strain Scott A grew in milk and media, but populations remained static in meat samples.

Listeria monocytogenes, an intracellular animal and human pathogen, has been implicated in several incidents of foodborne disease (*7*). These outbreaks were traced to consumption of contaminated milk, coleslaw, and soft cheeses (*12,13,21*). Examination of food products has indicated its presence in dairy products and meats (*18,19*), although limited in market vegetables (*20*).

High iron levels are often a prime factor in microbial virulence (*9,11*), and meat products might be expected to be potential substrates for virulent *L. monocytogenes* in the food chain. Beef chuck, for example, contains 0.45 mmole iron and 1.5 mmoles calcium per kg separable lean tissue. Skim milk, however, contains only 0.9 micromole iron but 30.75 millimoles calcium per kg (*1*). For this organism, potential virulence factors related to iron content are hemolysin and the oxidative enzymes, catalase, peroxidase, and superoxide dismutase (*25*). We have examined the role of iron levels in sterile milk, meat, and various media on the production of hemolysin and catalase by an isolated foodborne strain (Scott A) under various conditions.

Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

MATERIALS AND METHODS

Chemicals

Bovine liver catalase and streptomycin sulfate were purchased from Sigma Chemical Co., St. Louis, MO. Bathophenanthroline, isopentyl alcohol, O-dianisidine, potassium ferricyanide, and p-phenylenediamine dihydrochloride were purchased from Eastman Kodak Co., Rochester, NY. Acrylamide, bis (N,N'-methylene bis-acrylamide), ammonium persulfate, TEMED, and the silver stain kit were purchased from Bio Rad Laboratories, Rockville Center, NY.

Growth media and organism

Tryptose phosphate broth (TPB) and agar, tryptic soy broth, and brain heart infusion broth media was purchased from Difco Laboratories, Detroit, MI. Meat (beef chucks) was purchased from commercial sources, outer surfaces removed aseptically, and ground with a sterilized grinder. Samples were vacuum packaged individually in plastic barrier bags, frozen at -20°C, irradiated (2 Mrad with Cs 137 irradiation source), and stored at -70°C until used. Ultra high-temperature (UHT) pasteurized skim milk was purchased as needed from local sources and used without further treatment.

L. monocytogenes strain Scott A (serotype 4b) was obtained from the Food and Drug Administration, Cincinnati, OH, and was a fecal isolate from an infected human in the Massachusetts milk listeriosis outbreak (*6,12*). This organism was maintained on tryptose phosphate and brain heart infusion agar slants at 5°C.

Preparation of cell exracts

Cultured cells of *L. monocytogenes* (24 h at 37°C) were harvested by centrifugation (12,000 x g) and washed 2X with distilled water with centrifugation. Washed cells were disrupted with glass beads, and the supernatant fluid decanted after centrifugation. Nucleic acids were removed by precipitation with 1 mg streptomycin sulfate per mg protein, and the supernatant fluid fractionated with addition of ammonium sulfate. The 60-80% ammonium sulfate fraction was dialyzed, applied to a Sephadex G-150 column, and fractionated.

Milk and meat inoculations

Sterile meat and milk samples were inoculated with TPB cultures (24 h, 37°C) of the organism to an initial population of 10^4 CFU/g. Counts for milk samples represent $\log_{10}$ CFU/ml.

Counts for meat are the most probable numbers (MPN) as determined by enrichment and direct plating on modified Vogel Johnson agar (*8*).

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were conducted by the method of Laemmli (*17*) with a 5% stacking gel and a 7.5% resolving gel. SDS concentrations were 0.1% in the SDS-PAGE gels and samples. Selected fractions from the G-150 column separation were examined by PAGE followed by the ferricyanide staining procedure of Woodbury et al. (*28*) to identify catalase activity of the separated proteins on the gel and with Commassie blue and with silver stain to identify protein location. Milk and meat extracts, a bovine liver catalase sample, and molecular weight standards were also run on the gels with the test fractions.

Iron determination and reduced iron media

Iron content was determined colorimetrically with the reagent o-bathophenanthroline by the method of Van de Bogart and Beinert (*23*) following a sulfuric acid-hydrogen peroxide digestion. Results are reported as µmoles/L or µmoles/kg. Reduced iron media were prepared using the ion exchange resin Chelex 100 or calcium chloride precipitation by the procedures of Cowart, Marquardt, and Foster (*10*).

Catalase and hemolysin measurement

The catalase activity of the cell extract was measured by the spectrophotometric method of Beers and Sizer (*3*) on the supernatant fluid and on fractions from subsequent purifications. Results are reported as units/mg dry weight.

Hemolysin activity was determined by a modification of the method of Asao et al. (*2*). Samples were buffered with 0.01 M Tris-HCl containing 0.9% NaCl and 0.02 M cysteine- HCl, pH 5.5. Sheep red blood cells were added to a final concentration of 1%, incubated at 37°C for 1 h, then centrifuged to remove intact cells. Supernatants were analyzed spectrophotometrically at 540 nm, and hemolysin units (HU) were determined as 50% of the total lysis achieved by incubating blood cells followed by total disruption with distilled water.

Sterile and inoculated skim milk and meat samples were also examined for catalase activity using PAGE. With the inoculated milk samples, 50 ml were treated with detergent to solubilize proteins, centrifuged, and the supernatant fluid decanted. The supernatant fluid was treated as described above for catalase isolation, except the final G-150 column purification step was omitted. With the inoculated ground meat, 10 g were shaken for 5 min with 50 ml 0.05 M phosphate buffer, pH 7.0. The solid meat particles were pelleted and removed by low speed centrifugation. The supernatant fluid was filtered through coarse filter paper to remove fat particles and centrifuged in a microfuge to pellet bacteria. The resuspended pellet and supernatant were treated as described for the milk samples.

RESULTS AND DISCUSSION

The Scott A strain of *L. monocytogenes* grew well in TPB and tryptic soy broth. In inoculated TPB, catalase activity of agitated flasks (120 rpm at either 22°C or 37°C) was almost 5X (3.9 vs 0.8 units/mg dry weight cells) that in static flasks (Table 1). Highest catalase activities were noted in broths containing added iron at a 50 µmolar level. Treatments of TPB that altered ionic composition (calcium chloride or chelex) increased catalase activity in all static cultures, but in agitated cultures only in chelex treated and iron supplemented flasks. Catalase appears to be an induced enzyme in this organism in response to aeration. The hemolysin level in the TPB was independent of aeration. When the iron content in the broth was reduced by treatment with an ion exchange resin (Chelex 100) or with

TABLE 1. *Growth, catalase, and hemolysin activities of* L. monocytogenes *cultured in agitated, static, iron depleted, iron added, and normal tryptose phosphate broth (TPB).*

Treatment	Total dry weight (DW) (mg)		Catalase (U/mg DW)		Hemolysin Hu/mg DW		Iron content (µM)	
	A	S	A	S	A	S	A	S
TPB	110	60	3.87	0.83	2.28	2.48	13.0	12.5
$CaCl_2$								
TREATED	75	50	1.12	4.92	0.81	2.36	1.5	1.0
"+ ions + Fe	100	50	82.14	3.63	2.91	4.20	45.0	45.0
"+ ions - Fe	50	40	1.76	4.92	0.94	4.25	4.2	5.6
Chelex								
TREATED	20	5	15.71	3.37	1.05	8.00	0.2	1.3
"+ ions + Fe	80	45	34.23	10.56	4.38	7.22	39.5	52.0
"+ ions - Fe	20	40	0.02	10.42	1.80	3.15	0.4	0.6

A = Agitated culture conditions; S = Static.

Catalase activity is determined by the method of Beers and Sizer (3). Hemolysin activity is expressed as hemolysin units (HU), where 1 HU is the amount required for 50% lysis of sheep red blood cells.

Cations were added back to depleted media to supply the following: Manganese 2.5 µM; Copper 2.0 µM; Cobalt 1.8 µM; Magnesium 1.0 mM, and Iron (when added) 50µM. Calcium was added only to the Chelex treated medium to a 1.0 mM level.

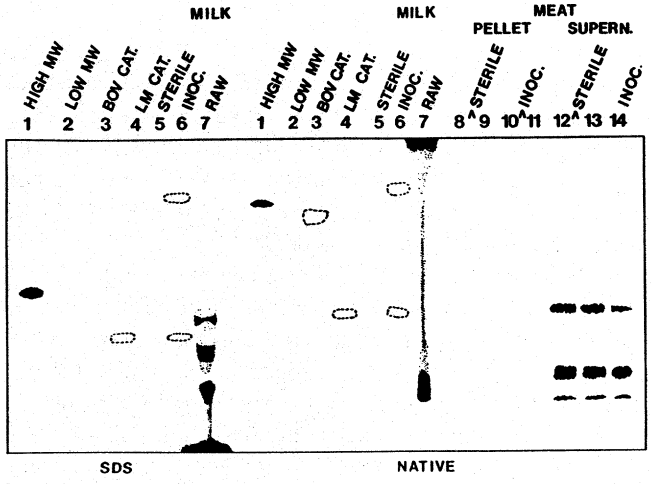

Figure 1. *SDS-PAGE and native PAGE electropherograms stained with ferricyanide to visualize catalase activity for partially purified catalase from* Listeria monocytogenes *(lane #4), milk sample (lanes #5-7), and meat (lanes #8-14) samples. Catalase activity is expressed as a bleaching of the background (dotted line areas). Raw whole milk (lane #7) but not sterile or inoculated skim milk (lanes # 5 & 6) contains components that react with the ferricyanide reagent to produce darkening. Minor darkening is also noted with meat supernatants (lanes #12-14) and the high MW standard myosin (lane #1).*

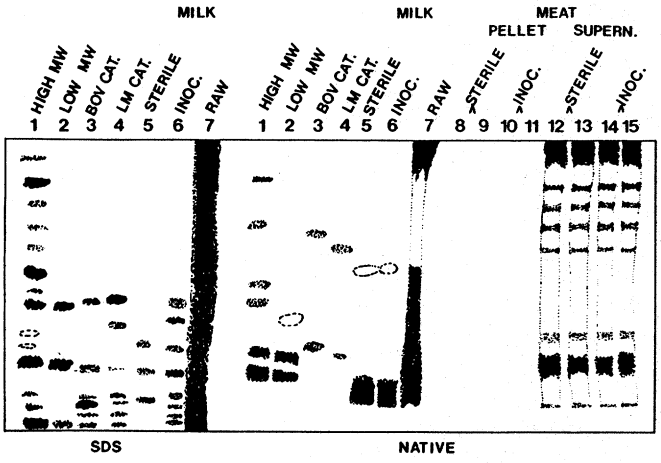

Figure 2. *SDS-PAGE and native PAGE electropherograms stained using silver stain to visualize proteins and catalase from* Listeria monocytogenes *(lane #4), milk (lanes #5-7), and meat (lanes #8-15).*

calcium chloride (*10*) before inoculation, there was an up to 92% decrease in growth as determined by dry weight. Selected ions were added back to the depleted medium before inoculation to measure the contribution of iron to this decreased growth and effect on catalase and hemolysin production. The ions and final levels in the medium were: Manganese -2.5 µM; copper - 2.0 µM; cobalt -1.8 µM; zinc - 1.8 µM; and magnesium - 1.0 mM. Calcium was added only to the Chelex treated medium to a 1.0 mM level. Iron was added where needed as the ferric chloride salt at a level of 50 µM (versus the level of ca. 13 M in the unextracted broth). Linear correlation coefficients of the iron content versus dry weight, catalase activity, or hemolysin activity were significant (P = 0.05) only in the agitated samples for catalase activity (r = 0.876) and hemolysin activity (r = 0.864) (*22*). Hemolysin activity, however, was consistently greater in static cultures than in aerated cultures at the same iron levels for reasons unknown.

PAGE of the extracted cellular material from TPB cultures following disruption by glass beads, ammonium sulfate fractionation, and Sepahadex G-150 column separation showed the presence of a major catalase band (MW of ca. 60 K daltons) and one or more minor bands (MW of ca. 200 K daltons) (Fig. 1 & 2). Interestingly, these bands from the cellular material remained stable when electrophoresed in the presence of sodium dodecyl sulfate, whereas bovine liver catalase did not (Fig. 2). This observation has also been reported by Wayne and Diaz (*24*). Purified extracts from *L. monocytogenes* Scott A cultivated in UHT milk also demonstrated catalase activity consistent with that observed for cultures cultivated in TPB. No catalase activity was detected for *L. monocytogenes* Scott A cultivated in meat. This may be a result of low microbial counts in the meat samples. After 5 months storage at 5°C post-inoculation with *L. monocytogenes* strain Scott A (initial CFU of 10^4/g), milk had a CFU of log value 8.65/ml, whereas the meat sample had a CFU of log value 3.23/g. The iron content for the meat was 0.5 mmoles/kg, and that of the milk samples ranged from 10.6-15.0 µmoles/L.

The virulence of *L. monocytogenes* has been attributed in part to the production of hemolysin (*5*) which apparently permits the organism to invade cells within the intestine similar to *Salmonella* and certain other foodborne organisms. Nonhemolytic *Listeria* are not virulent in humans, and transposon mutants of *L. monocytogenes* lacking production of hemolysin were avirulent (*14,15*). Catalase and superoxide dismutase (*9*) have been implicated as virulence factors in that these enzymes may protect the organism from the oxidative bursts of peroxide and other active oxygen species while engulfed by phagocytes. Catalases are metalloenzymes, usually containing iron (*9*). Taxonomically, *Listeria* spp. are closely related to *Lactobacillus* spp. (*26*), from which noniron catalases containing manganese have been isolated (*16*).

Both catalase and hemolysin have been related to iron levels, in that hemolysin apparently is produced in low iron media, and catalase is dependent upon iron in the medium. In these experiments (*4,14*), the degree of aeration was more a factor in both growth and in catalase activity, although iron level was an interactive component. Iron is essential for high catalase activity, and the presence of manganese and other cations had little effect. With hemolysin, as with catalase, there was a relationship with iron content, although it was opposite to the expected increase with low iron levels. Hemolysin appears to be produced more in static cultures than aerated cultures, for reasons unknown. Although only intracellular levels of catalase and hemolysin were examined as a function of iron concentration, an additional (or alternative) effect of iron may be upon the transport of these factors across the bacterial cell membrane.

The reason for the poor growth found for strain Scott A in meat at refrigerator temperatures requires further study. Although it appears to be the most common serotype in human illness isolates, the serotype (4*b*) has seldom been found in meat samples positive for *Listeria (27)* and apparently does not grow or die off during storage if inoculated into meat.

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